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UNDERSTANDING AMYLOSE SYNTHESIS TO DEVELOP HIGH AMYLOSE WHEAT

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Understanding Amylose Synthesis to Develop High Amylose Wheat Final Report – April 01, 2003 to October 31, 2006

Principal Investigator : Ravindra Chibbar

Co-investigators: Pierre Hucl and Monica Baga

Department of Plant Sciences/ Crop Development Centre, University of Saskatchewan.

51 Campus Drive, Saskatoon, Saskatchewan S7N 5A8

(a) Abstract/Summary: Wheat lines with amylose concentrations ranging from 32-34.5% were produced. The high amylose lines were obtained by the sequential elimination from starch granules of starch synthase II, as determined by denaturing gel electrophoresis. Chemical composition of high amylose starch was similar to that of normal wheat starch, except that total starch yield was low in a high amylose line studied. Starch gelatinization parameters, onset, peak and conclusion temperature of gelatinization was lower than normal wheat starch. Increase in wheat grain starch amylose concentration also changed gelatinization parameters of extracted starch.

(b) Executive Summary

The main objective of this project was to increase the wheat grain amylose concentration. By sequential elimination of starch synthase II from A and B genomes, amylose concentration was increased to more than 32%, as compared to 27% in normal wheat starch. The five percent increase is significant, because it does change the starch gelatinization properties. Wheat lines with increased amylose concentration should now be tested for performance in baking and/or noodle making. However, research should still be continued to increase amylose concentrations more than 32% that has been achieved in this project. Amylose concentrations as high as 79% is possible as it has been recently shown by transgenic wheat in which starch branching enzyme II had been eliminated by RNAi technology. As transgenic wheat is still not acceptable to customers of Canadian wheat, non-GMO strategies should be pursued to increase amylose concentration. One of the strategies is to screen wheat Targeted Induced Local Lesions in Genome (TILLING) population to identify wheat lines with null alleles for starch branching enzymes or other starch synthases such as starch synthase I and/or III. The identified lines should be characterized for starch biosynthetic enzymes and after confirmation should be back crossed to wheat lines with SSII null alleles produced in this project to develop wheat lines with increased amylose concentration.

(c) Technical Report

Introduction and Objectives: Cereal grains contribute one-half of the calories in the human diet. Most of this energy is derived from carbohydrates, which account for 70-80% of grain dry weight (Chibbar et al, 2004). Carbohydrates are also a renewable industrial raw material, with hundreds of applications from fillers in detergents to biodegradable staples used in human surgical procedures (Davis et al., 2003; Jobling 2004). Carbohydrates

are classified in several ways, depending on their chemical structure, spatial occurrence in plants or their utilization by humans and animals (Chibbar and Båga, 2003; Chibbar et al., 2004). Chemically, simple carbohydrates are composed of one to two hexose or pentose molecules, oligosaccharides consist of three to twenty molecules, and complex carbohydrates are made up of twenty to several thousand molecules chemically linked together. Complex carbohydrates are mainly present as structural carbohydrates (cellulose, xylose, callose) and storage carbohydrates (for example starch, xylans, beta-glucans), in plants.

Starch structure: Starch is the major reserve carbohydrate in cereal grains including wheat (Chibbar et al., 2004). Starch is a complex macromolecule composed of two glucan polymers, an essentially linear chain amylose and a highly branched amylopectin. Amylose (molecular mass 10^5 to 10^6 Da) is composed of linear chains of a-D-glucopyranosyl residues linked by α-(l→ 4) bonds, with a few molecules (0.5%) branched by α-(l→ 6) glucosidic linkages. Amylopectin (molecular mass 10^7 to 10^9 Da) is highly branched with 4% to 5% of the α-(l→ 6) glucosidic linkages. Starch is normally made up of one-quarter amylose and three-quarters amylopectin. Both the proportion of amylose to amylopectin and structure of amylose and amylopectin molecules vary with the plant species, thus giving a natural diversity of starch molecules. Starch occurs in plants with amylose and amylopectin arranged in a three-dimensional semicrystalline structure - the starch granule. In addition to the glucan polymers, the starch granule contains small amounts of lipids (0.1 - 1.0%), proteins (0.05 - 0.5%) and trace amounts of phosphates (Smith et al., 1997).

Starch biosynthesis: Biosynthesis of starch in plants has been actively studied. Several recent reviews have summarized the extensive data related to starch biosynthesis (Ball and Morell, 2003; Jobling 2004; Smith et al., 1997). Studies of starch mutants have so far produced a general consensus that ADP-glucose pyrophosphorylase (AGPase), soluble starch synthases (SS), starch branching enzymes (SBE), starch debranching enzymes (DBE; pullulanase; isoamylase) and possibly also disproportionating enzyme (D-enzyme) catalyze the final steps leading to amylopectin synthesis. Granule bound starch synthase 1 (GBSS 1) is the only enzyme committed to amylose synthesis. Several of the enzymes exist in different isoforms, some of which vary in their sub-cellular distribution, enzyme specificity, temporal activity and interaction with other enzymes, thus making starch synthesis pathway very complex. A minimal subset of 14 conserved starch biosynthetic enzymes (two AGPases, five SS, three SBE and four DBE) is homologous in all the plant species studied to-date (Morell and Myers 2005). Some of these enzymes have specific functions in starch biosynthesis. However, the priming mechanism for starch glucan polymerization in plants is unclear, but it likely involves short-chain maltodextrins that are extended and branched by the action of GBSS1, SBE1 and SS1 to form a molecule with amylopectin-like backbone. The primary glucan polymer is further polymerized through cycles of chain extensions and branching catalyzed mainly by SSI and SBEII. Besides the core enzymes (Morell and Myers, 2005), additional enzymes such as starch phosphorylases, disproportionating enzymes and glucan water kinases also play important roles in starch biosynthesis. Recent studies suggest post-translational modifications of the core starch biosynthetic enzymes, which introduces a new level of complexity in our attempts to modify starch structure *in plant a*. However, a major objective of my research program is to understand the molecular mechanism of starch biosynthesis in wheat and barley.

High amylose starch: Starch with elevated amylose concentration (>40%) is preferred for gums and candies and in the pulp industry. Recently, an increased demand for high amylose wheat starch within the food industry has emerged as high-amylose starch can be converted to resistant starch (RS) upon heating and subsequent cooling. RS is not digested in the small intestine, but is broken down by the bacteria in the colon. As a result, RS acts as dietary fiber (DF), it reduces the calories from food, has low glycemic index and is considered beneficial for colon health. An increase in amylose/amylopectin ratio and higher average amylopectin branch length in starch is found in certain maize, rice and pea mutants, which lack expression of one of the two SBEII isoforms (Chibbar and Båga, 2003). The high amylose mutants in maize and pea have amylose concentrations of 50% or greater as compared to 25-30% for the normal endosperm and embryo starches. In mutant indica rice, the amylose content is 39 - 41% compared to 24-25% for the wild type (Kaushik and Khush 1991). High amylose barley lines with grain endosperm amylose concentration up to 40% have been developed (B.G. Rossnagel, personal communication). The amylose fraction in some mutants has a lower molecular weight and wider size distribution than normal amylose. In high amylose barley (Himalaya) the mutation has been attributed to SSIIa deficiency (Morell et al., 2003). Similarly, elimination of SSII polypeptides in wheat increased apparent amylose (colorimetric assays) concentrations to 30-37% (Yamamori et al., 2000). In a similar approach, we have developed wheat lines deficient in SSII A and B genome polypeptides, which resulted in lines showing up to 35% amylose as determined by HP-SEC analyses (Chibbar et al., 2005).

The main objective of this project was to develop and characterize wheat lines with high amylose. The main objective was divided in to three sub-objectives, which also formed the yearly milestones under which progress was reported. The technical report will be summarized under three subheadings.

1. Development of near-homozygous (NH) **lines from high-amylose wheat segregants:**

In the year 2003, nearly 8,000 hill plots of experimental wheat were grown for this project at the University of Saskatchewan's Seed Farm with partial irrigation. Several groups of material were grown out. The first group consisted of 2,150 hills of lines in the F_6 and F_7 generations. These lines were from crosses designed to stack individual starch synthase mutations. The parents were generally winter or facultative genotypes. The 55 populations were screened for the ability to produce mature seed by late-seeding the nursery in 2001 and 2002 thus maximizing vernalization effects. Approximately 30% of this material was harvested for re-evaluation in 2004. Selected lines in paired hills were grown in 2004 and their amylose concentrations were determined. The second group of lines (F_4 and F_5 generations) was from crosses between stacked genotypes and a Canadian spring wheat parent. In excess of 2,400 lines were in this group. This material was earlier maturing than the primary germplasm used as a source of SS II variation. The cull rate on this material was roughly 50%. The third group of lines consisted of tetraploid populations in the F_4 and F_5 generations. There were 3,200 lines in this group. The cull rate was only 25%. The major selection criterion was tetraploid spike morphology. The objective was to screen some of the most promising populations for amylose concentration prior to the 2004 planting season. Some of the lines were in the null A and B state and this resulted in

higher amylose levels in tetraploid wheat. In addition to the 8,000 genotypes grown in hill plots in 2003, 37 tetraploid populations were grown as F_2 bulks. This material was grown as F_3 bulks in 2004.

Based on our results from the first year (2003) of the project, we focused our efforts in introgressing SSII null alleles in to hexaploid and tetraploid spring wheat cultivars grown in Western Canada. Crosses were made during the course of the project to introgress SSII null alleles in to hexaploid and tetraploid spring wheat backgrounds. Initially SSII null alleles (A and B) were backcrossed in to hexaploid wheats CDC Rama, AC Superb, 5700PR and durum AC Avonlea and AC Navigator. 1770 seeds (870 -durum and 900 -hexaploid) wheat lines belonging to 403 families (103-durum and 300 - hexaploid) were analyzed for amylose concentration using HPSEC as described before (Demeke et al 1999). 197 seeds (150 durum and 47 hexaploid) showed amylose concentrations higher than 30%. Some of the durum lines showed high degree of homozygosity in amylose concentrations. Sixty four selected lines that showed consistently high amylose concentration were sent to winter nursery for seed increase.

In another experiment the SSII null alleles have also been backcrossed in to a durum (AC Avonlea) and bread wheat (AC Superb). The F_7 seeds were harvested and these were analyzed for amylose concentration with HPSEC.

Identification of high-amylose wheat lines: In the year 3 of the project, grain from sixty four selected lines that showed consistently high amylose concentration was received from winter nursery in New Zealand. Starch was extracted from grain samples and analyzed for amylose concentration. Results presented in Figure 1 show several lines with amylose concentrations from 30% to 35% have been developed. The control checks had a grain amylose concentration of 25 to 26%.

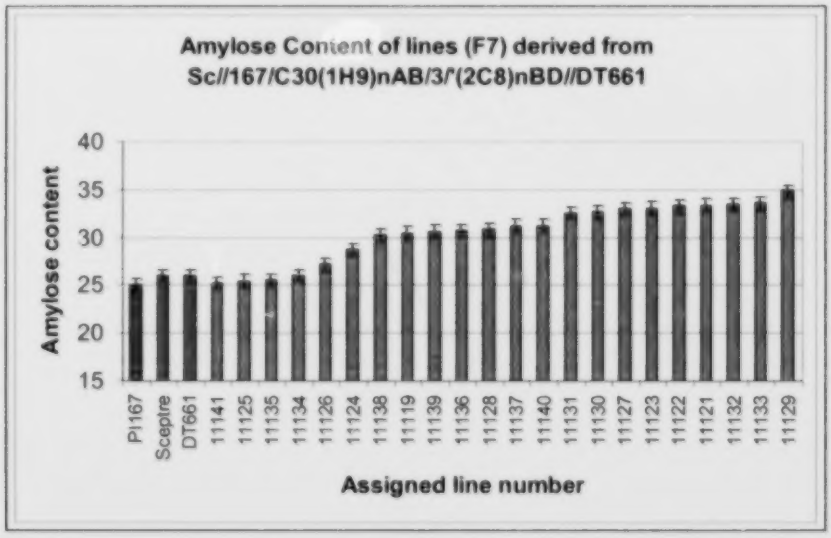


Figure 1. Amylose concentrations of selected wheat lines. PI167, Sceptre and DT661 are the three parents. The assigned line numbers denote the wheat lines developed during this project.

The wheat lines that showed amylose concentrations higher than 32% were also field tested in Saskatoon. The grain was used to extract starch by a wet-milling procedure for analysis of chemical properties and starch gelatinization studies.

2. Biochemical and molecular characterization of wheat segregants for *SsII*: To assess the presence of SSII in wheat segregants, the grain was split into half, with the embryo portion being retained for growing, and the non-embryo portion was used to extract starch. We used a denaturing gel electrophoresis technique to assess the presence of SSII alleles in grain starch.

Isolation of starch granules: Starch was isolated from the embryo-less half of the wheat grain essentially using a procedure described by Zhao and Sharp (1996) and Demeke et al (1997, 1999). The embryo-less half was cut in to several small pieces and placed in 1 mL of distilled sterile water in a microfuge tube (1.5 mL) kept overnight at 4°C. The water was carefully decanted and a small pestle was used to grind the seed into slurry. The slurry was layered over 1 mL of Cesium chloride (80%) in a 2 mL microfuge tube. The microfuge tube was centrifuged at 13000g for 5 minutes. The supernatant was discarded and the starch granules pelleted at the bottom were centrifuged once again through cesium chloride to remove any adhering non-starch molecules. The pelleted starch granules were washed twice with wash buffer (55 mM Tris-HCl pH 6.8, 2.3 % SDS, 10 % glycerol), once with water and finally with acetone. After the final wash, the starch granules were dried and used for SSII analysis by gel electrophoresis.

Extraction of Starch Granule Proteins: 10 mg starch granules were suspended in 150 uL of sample buffer (62.5 mM Tris-HCl pH 6.8, 10% SDS, 10% glycerol, 0.0005% bromophenol blue, 5% β-mercaptoethanol) and treated in a boiling water bath for five minutes. After boiling, the tube was immediately cooled for 5 min on ice to allow the starch to gel. The gelled starch solution was centrifuged at 15,000g for 12 minutes and the supernatant contained the starch granule proteins.

Electrophoretic (SDS-PAGE) separation of starch granule proteins: To analyze SSII proteins, starch granule bound proteins were separated by denaturing discontinuous gel electrophoresis. The separating gel contained 10% polyacrylamide, 0.135% bisacrylamide, 0.375 M Tris-Hcl (pH 8.8), 0.1% SDS, 0.05% ammonium persulphate, and 0.05% TEMED. Stacking gel contained 4% polyacrylamide, 0.8% bisacrylamide, 126 mM Tris-HCl, 0.05% ammonium persulphate, and 0.05% TEMED. The running buffer contained 10 g SDS, 30.28g Tris salt, 144.2g Glycine made to 1L water containing 0.1% β -mercaptoethanol. The proteins were electrophoresed for 225 mA hours. After electrophoresis, proteins were visualized by silver staining using a commercial kit Silver Stain Plus (BioRad) as per the manufacturer's instructions.

Amylose determination: A high performance size-exclusion chromatography (HP-SEC) method was used to determine amylose concentration (Demeke et al., 1999). 5 mg starch sample was suspended in 5 mL distilled water in a glass tube and incubated at 130°C for 30 min. To one mL of gelatinized starch solution mixed vigorously, 55 uL of 1M sodium acetate (pH 4.0) was added and mixed vigorously. Four units of isoamylase was added to the starch solution to de-branch starch. After four hours of incubation at 40°C, the debranching reaction was stopped by boiling for 20 min to inactivate isoamylase. The debranched starch solution

was freeze dried. The freeze-dried sample was dissolved in 200µL of DMSO (99% v/v) and then centrifuged in a microfuge 15,000 g.min. Supernatant (40uL) was injected in to a PLgel MiniMix-C guard column attached to a PLgel MiniMix 4.6 mm i.d. column (Polymer Laboratories, Inc. Amherst, MA) to separate amylose and amylopectin using an HPLC system (Waters 600 controller, Waters 610 fluid unit, Waters 717 plus autosampler, Waters 410 differential refractometer). The data were collected and analyzed using Millenium software. Starch samples, column, and detector were maintained at 40, 100 and 45°C, respectively. DMSO (99%) was used as an eluent at a flow rate of 0.2 mL/min. The amylose concentration was calculated by integration of the peak area corresponding to amylose to that of the peak area corresponding to both amylose and amylopectin.

Identification of wheat SSII null lines: Denaturing gel electrophoresis of starch granule proteins could separate SSII, A, B and D genome products (Figure 2). The SSII B genome product showed the highest mobility while A genome SSII showed the least mobility. This technique was used to screen all the material produced in section 1. A total of more than two thousand lines were screened during the course of the project. A SDS-PAGE silver stained gel is shown in Figure 2.

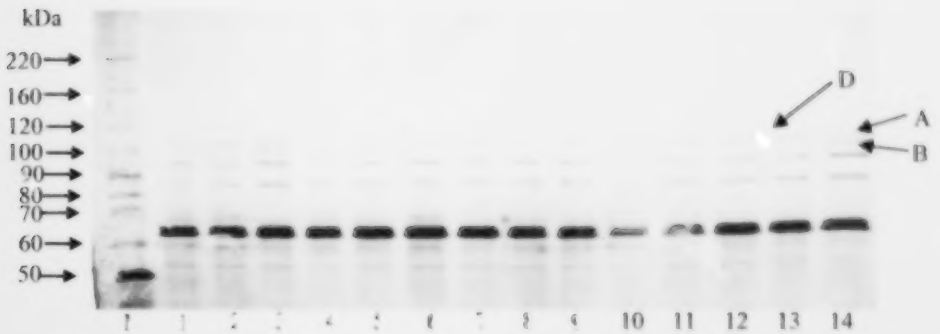
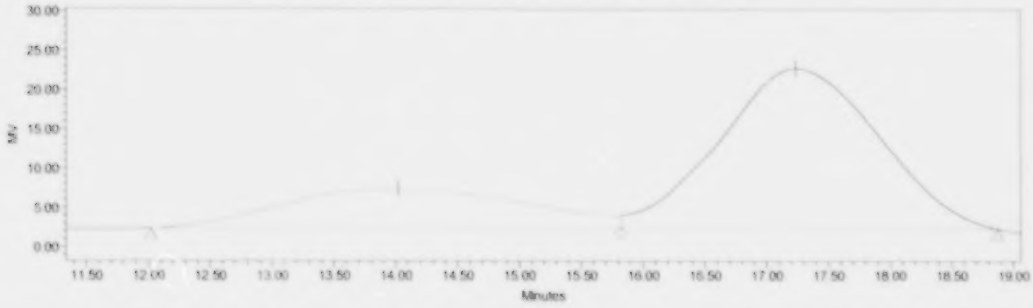


Figure 2. SDS-PAGE separation of starch granule bound proteins to show the separation of SS II, A, B and D genomes (arrows) of wheat. The gel has been silver stained to visualize the proteins. **Lane 1,** 36-25 1E; **Lane 2**, 36-25 1F; **Lane** 3, Barrie; **Lane 4,** 36-25 1G; **Lane 5,** 36-25 1H; **Lane 6**, 36-25 2A; **Lane 7**, 36-25 2B; **Lane 8**, 36-25 2C; **Lane 9**, 36-25 2E; **Lane 10**, Andrew; **Lane 11**, 36-25 2F; **Lane 12** 36-25 2G; **Lane 13**, 36-25 2F; **Lane 14**, 132 4B. The A, B and D show SSII from the three wheat genomes, respectively. **Lane M,** Molecular weight of Protein marker (Invitrogen) ranging from 220kDa to 50kDa.

Determination of Amylose concentration: The HPLC separation method used in our laboratory could consistently separate amylose from amylopectin (Figure 3).

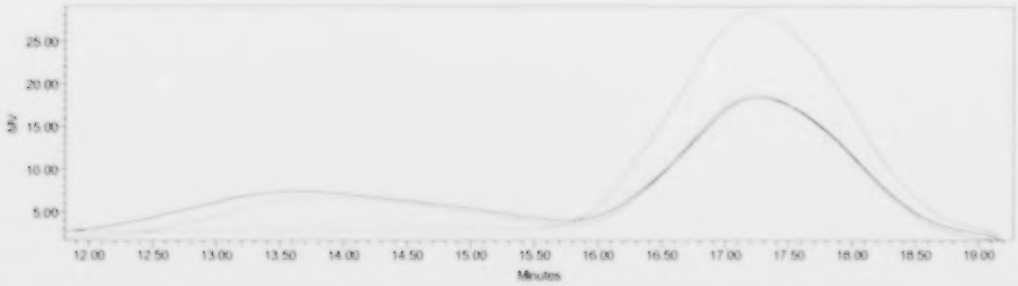
Screening of wheat segregants and pure-lines for amylose concentration using HPLC-SEC analysis. Starch extracted from several thousand embryo-less half seeds was analyzed for amylose and amylopectin concentrations. Figure 4 shows the amylose and amylopectin concentration in a normal hexaploid wheat, identified high-amylose wheat line and a waxy wheat line. The high-amylose wheat lines show a slight increase in peak height at the early elution time, which is an indication of increase in amylose molecular weight. Amylose with higher molecular weight is

suggested to have more beneficial effects in terms of resistant starch development. A distinct advantage of the HPLC method is the ability to get some indication of the size of the molecule being separated. In addition, a review of several hundred HPLC chromatograms revealed changes both in amylose and amylopectin structures of the wheat lines developed in this project. However, for this project only a few lines were selected.



	Name	Retention Time	Area	% Area	Height	Start Time	End Time	Baseline Start	Baseline End
1	Amylose	14.021	678177	27.16	4939	12.017	15.817	12.017	18.867
2	Amylopectin	17.238	1819080	72.84	20388	15.817	18.867	12.017	18.867

Figure 3: A representative HPLC chromatogram to show the separation of amylose (red) and amylopectin (blue). The table shows the integration of peak areas, which represents relative concentrations of amylose and amylopectin.



Sample	Component	Retention Time	% Area	Start Time	End Time	Baseline Start	Baseline End	Remarks
CDC Teal	Amylose	13.542	25.93	11.633	15.717	11.633	18.983	Normal wheat
	Amylopectin	17.28	74.07	15.717	18.983	11.633	18.983	
132 28C	Amylose	13.624	31.84	11.783	15.817	11.783	18.95	High amylose
	Amylopectin	17.233	68.16	15.817	18.95	11.783	18.95	
99 Waxy 27	Amylopecin	17.218	100	15.45	19.017	15.45	19.017	Waxy line

Figure 4: A HPLC chromatogram to show amylose and amylopectin separation in CDC Teal (green line), high amylose wheat line -11132-28C (black line) and a waxy wheat line 99Waxy-27 (red line).

Starch extracted from more than two thousand wheat lines was analysed by SDS-PAGE for SSII alleles and HPLC for amylose concentrations showed that deficiency of SSII alleles resulted in increase of amylose concentration (Table 1). Wheat lines deficient in SSII A or B, or SSII A and B together could be detected. However, we could not detect a wheat line which was deficient in both SSII B and D or all the three SSII alleles (Table 1). Amylose concentrations could be increased to more than 32%. Total starch in the SSII deficient lines was in the range of 53 to 71%, which is expected for such material. Except for one line, protein concentration was also within the expected limits.

Table 1. Grain starch amylose concentrations in wheat lines deficient in SSII

Genotype	% Amylose range	% Total Starch range	% Total Protein range
Normal	25.01 to 28.13	59.10 to 66.80	11.00 to 15.97
null A	27.06 to 30.93	57.60 to 67.80	11.53 to 18.63
null B	28.08 to 30.34	65.80 to 67.30	12.76 to 12.97
null D	26.77 to 29.69	62.90 to 70.60	13.65 to 16.15
nullA, nullB	25.60 to 32.69	52.70 to 71.10	11.82 to 22.71
nullA, nullD	27.86 to 31.84	60.00 to 75.00	13.08 to 15.90

3. Determination of starch properties: For the study of starch properties, large scale starch extraction was done by a wet milling process as described below.

Starch Extraction: Wheat grain (200g) was mixed with 500 mL sodium bisulfite (0.5%) at 4°C for 16hr. After steeping the wheat was drained and rinsed with distilled water to remove any sodium bisulfite. For every 100 g of wheat 200 mL of water was added and blended for two min at high speed in a Waring blender. The homogenate was passed through 210 µm polypropylene screen or a cheese cloth. The residue on the screen was homogenized twice with 100 mL water and filtered as before. The combined filtrate was allowed to sediment overnight at 4°C. The supernatant was discarded and the starch slurry was suspended in 200 mL of water. The pH of the starch solution was adjusted to pH 9.0 with 1.0 N sodium hydroxide with continuous agitation. The alkaline starch suspension was centrifuged for 45,000g min. The top proteinaceous layer was scraped off and this step was repeated twice. The starch was resuspended in 200 mL water, and the pH was brought to 7.0 with 0.5 N HC1. The starch slurry was centrifuged for 45,000 g.min. The pelleted starch was washed with water in a Buchner funnel lined with a #4 Whatman filter paper. The final rinse was with ethanol (95%). The starch was dried overnight and weighed to calculate starch obtained.

Starch Granule Size Determination: The sizes of starch granules from the three wheat lines were analyzed by the following method. Starch granules were suspended in 90% (v/v) ethanol. A drop of the starch suspension was spread on a microscope slide and air-dried. The slide was then placed on the stage of a research microscope (Zeiss). Images of starch granules were

analyzed using an image analyzer that was equipped with image acquisition and processing software (Northern Eclipse 6.0, Empix Imaging Inc. Mississauga, Ontario, Canada). More than 1000 starch granules from each of the starch granule populations were analyzed. Starch granules were grouped according to their diameters, and the number of starch granules in each group was counted. Plotting the relative number of starch granules against granule diameters produced a starch granule size distribution curve. From this curve the percentage A and B -type starch granules were calculated.

Proximate analysis: Quantitative estimation of moisture, ash and nitrogen content was determined by the standard AACC (2000) methods. Starch lipids (surface and bound) were determined as described (Waduge et al., 2006).

Apparent amylose content: Starch (20 mg) was suspended in distilled water (6 mL) in glass boiling tubes and mixed vigorously. Two mL of sodium hydroxide (1M) was added to the starch slurry and mixed vigorously and then heated in a water bath (85°C) for 15 min. After cooling to room temperature the starch solution was made up to 25mL in a volumetric flask. Two mL of diluted starch solution was mixed 45 ml of phosphate buffer (0.06 M, pH 8.0) and 1 mL of I_2/KI solution (0.005 M I_2 and 0.018M KI). The final volume was adjusted to 50 mL in a volumetric flask, and allowed to develop color for 30 minutes at room temperature in dark. The absorbance of the resulting blue color was measured at 620nm in a spectrophotometer. Total amylose content was also measured by the above procedure but with prior de-fatting with hot n-propanol-water (3:1 v/v) for seven hours. The total amylose content was calculated from a standard curve using mixtures of pure potato amylose and amylopectin, over the range of 0 to 100%.

Starch phosphorous: Total starch phosphorous was determined essentially as described before (Wagude et al., 2006). Briefly, 5 mg starch was placed in glass tube and gently heated with 0.3 mL concentrated sulfuric acid until charring was completed. After cooling to room temperature, hydrogen peroxide (20 uL, 30% w/v) was added to hit the walls of the tube just above the acid, and the tubes were well shaken. The tube was gently boiled for 30seconds and the solution was allowed to cool to room temperature. The volume was made up to 3.6 mL with distilled water. To assay, sodium sulfite (0.1 mL, 33% w/v) was added with stirring followed by addition of ammonium paramolybdate (0.1 mL, 2% w/v) and ascorbic acid (0.01 g). The contents of the tubes were vigorously mixed by vortexing and then heated for 10 minutes in a boiling water bath. After cooling the tubes to room temperature, the final volume was made to 5 mL with distilled water, and the absorbance was read at 822 nm using a spectrophotometer.

Differential scanning calorimetry was used to study the starch gelatinization and retrogradation characteristics as described (Waduge et al., 2006). Starch pasting properties were analyzed by Rapid Visco™ Analyser RV-4 (Newport Scientific Pty, Ltd, Warriewood, NSW, Australia) as described by Waduge et al (2006). Methods to study starch swelling factor, amylose leaching, acid hydrolysis and enzymatic digestibility have also been recently described (Waduge et al., 2006).

Results: Three lines, 11132, CDC Teal and 99WAX27 were selected for detailed analyses of starch properties. Line 11132 was selected from a group of lines derived from the same cross and consistently showed 32% or more amylose concentration. CDC Teal was selected as a normal bread wheat control, and 99 WAX27 is a line, which has no amylose, but more than 99% amylopectin (Demeke et al., 1999). The

Table 2: Chemical composition of native starches isolated by wet milling from the three selected wheat lines.

Characteristics (%)	Starch Source (Genotypes / Lines)		
	CDC Teal	11132	99WAX27
Starch yield (based on original seed weight)	43.6±2.0	32.5±2.5	48.0±2.2
Moisture	9.11±0.021[a]	9.27±0.021[b]	10.44±0.025[c]
Ash	0.15±0.01[d]	0.15±0[d]	0.03±0[e]
Lipid : solvent extracted			
Chloroform-methanol[1]	0.03±0.01[f]	0.07±0.02[g]	0.02±0.01[f]
n-propanol-water[2]	0.69±0.06[h]	0.77±0.05[h]	0.24±0.01[i]
Lysophospholipid (LPL)[3]	0.41	0.35	0.011
Free fatty acid (FFA)[4]	0.37	0.42	0.25
Amylose content			
Apparent[5]	23.28±0.11[j]	26.34±0.09[k]	0
Total[6]	26.87±0.21[l]	32.29±0.15[m]	0
Phosphorus content			
Non lipid	0.034±0[n]	0.033±0[o]	0.0068±0[p]
Total	0.056±0[q]	0.058±o[r]	0.0075±0[s]
Starch damage	Not detected	Not detected	Not detected

All data reported on dry basis and represent the mean of at least three replicates. Values followed by the same superscript in each row are not significantly different (P0.05) by Tukey's HSD test.

[1] Lipids extracted from native starch by chloroform-methanol (CM) 2:1 (v/v) at room temperature (mainly free lipids).

[2] Lipids extracted by hot 1-propanol-water (PW) 3:1 (v/v) from the residue left after CM extraction (mainly bound lipids).

[3] LPL=16.39 x (native starch phosphorous-defatted starch phosphorous)

[4] FFA=Total lipid-LPL

[5] Apparent amylose determined by iodine binding without removal of free and bound lipids.

[6] Total amylose determined by iodine binding after removal of free and bound lipids.

starch yield in 11132 was lower than CDC Teal and the waxy wheat line 99WAX27. Both the apparent amylose content and total amylose is higher in the wheat line 11132 as compared to CDC Teal. The most interesting observation is that the total amylose concentration determined by a colorimetric assay described above in the line 11132 and CDC Teal is precisely the same as determined by the HPLC method described above. In both cases, CDC Teal had 26% and line 11132 showed 32%. The results confirm that the grain amylose concentration has been increased in wheat line 11132. There was no significant difference in the starch granule size distribution between the three lines studied. As expected lipid content was slightly higher in 11132 as compared to CDC Teal, but the waxy line had considerably lower lipid content. Phosphorous is a minor starch component and it does not differ between CDC Teal and 11132, however, it is slightly higher in the waxy line.

Starch granule size determination: Tall the three wheat starches studied contained both A- and B-type starch granules (Table 3). In addition, the distribution of the two types of starch granules also did not vary significantly between the three lines, CDC Teal, 11132 and 99WAX27 (Table). The large A-type granules were highest in CDC Teal accounting for 8% of the total granules (numbers), while in the waxy starch A-type granules accounted for only 5% of the total granules. This observation concurs with earlier reports comparing waxy starch with normal wheat starch (Abdel-Aal, 2002). This shows that increase in amylose concentration does not affect starch granule size.

Table 3. Granule size distribution of the three native wheat starches

Genotype/Line	Granule size (%)	
	A-type (≥10μm)	B-type (≤10μm)
CDC Teal	8.8	91.2
11132	6.7	93.3
99WAX27	5.1	94.9

Swelling Factor (SF): The swelling factor of the three native starches heated in the temperature range of 50-90°C showed that waxy wheat line had the highest swelling factor followed by CDC Teal and the lowest in line 11132 (Figure 5). The decrease in swelling factor with increased amylose concentration has been shown in barley (Waduge et al., 2006), and maize (Jayakody and Hoover, 2002, Tester et al., 2000). As shown for barley and maize, in waxy wheat starch, the SF dramatically increased between 50 – 60°C, while for CDC Teal and line 11132, SF increased gradually between 60-80°C and rapidly increased between 80 – 90°C (Figure 5).

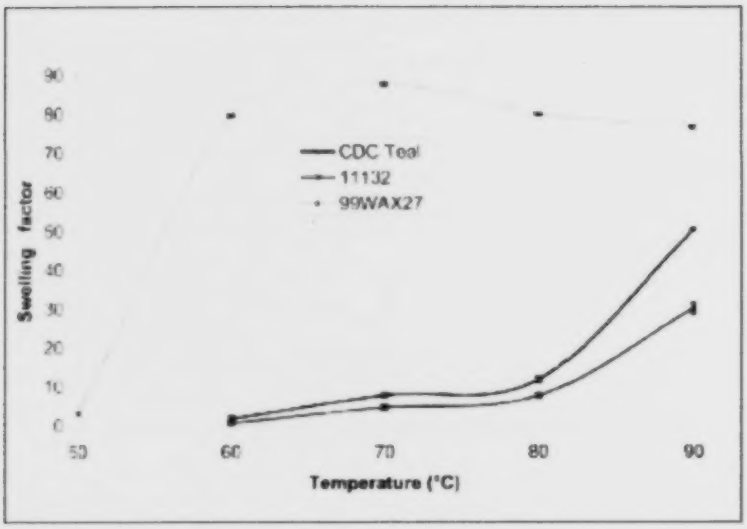


Figure 5. Swelling factor of the three native starches.

Amylose leaching: Amylose leaching from 70 to 80°C of native starch in both CDC Teal and 11132 increased gradually and at the same rate (Figure 6). However,

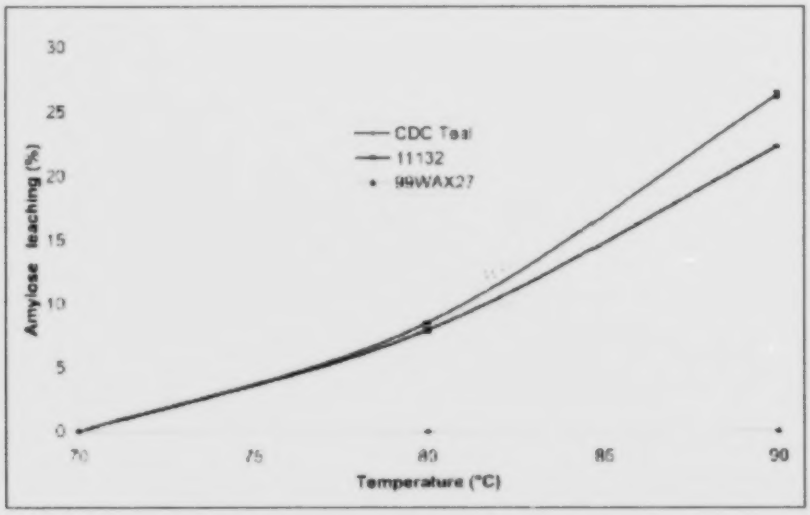


Figure 6. Amylose leaching in the two native starches in the temperature range 70 – 90°C. Note waxy starch has no amylose, therefore amylose leaching is not studied.

between 80 to 90°C the high amylose starch from wheat line 11132 showed slightly higher amylose leaching than CDC Teal. No amylose leaching was observed in the waxy wheat line, as only amylopectin is present in the starch. Amylose leaching of native starch is influenced by several factors, such as total amylose concentration, amount of lipid complexed with amylose chains and association of amylose chains with

each other or amylopectin (Hoover and Vasanthan, 1994; Nakazawa and Wang, 2003; Waduge et al., 2006). The slightly increased amylose leaching observed in 11132, further confirms that amylose concentration in this line has been increased.

Thermal characteristics: The starch gelatinization transition temperatures (onset, peak, and completion) and gelatinization temperature range of the high amylose line was lower than the normal wheat (CDC Teal) and waxy (99 WAX27) wheat line (Figure 7, Table 4). The normal wheat CDC Teal had the highest onset and peak temperatures but a lower completion temperature as compared to waxy wheat starch. Waxy wheat starch had the highest completion temperature and consequently a larger range as compared to CDC Teal and high amylose wheat starch. The results regarding the onset, peak and completion temperatures related to amylose concentration do not follow a definite trend. Abdel-Aal et al (2002) found very little difference in the onset, peak and completion temperatures and enthalpy of gelatinization between normal and waxy wheat starch, whereas in waxy corn and barley starch the energy of enthalpy was highest in waxy, followed by normal and lowest in high amylose starches (Waduge et al., 2006). Similar higher energy of enthalpy as compared to normal starch was also for waxy wheat starchhas been reported. The slightly lower energy of enthalpy in the line 11132 suggests that this line has higher amylose concentration.

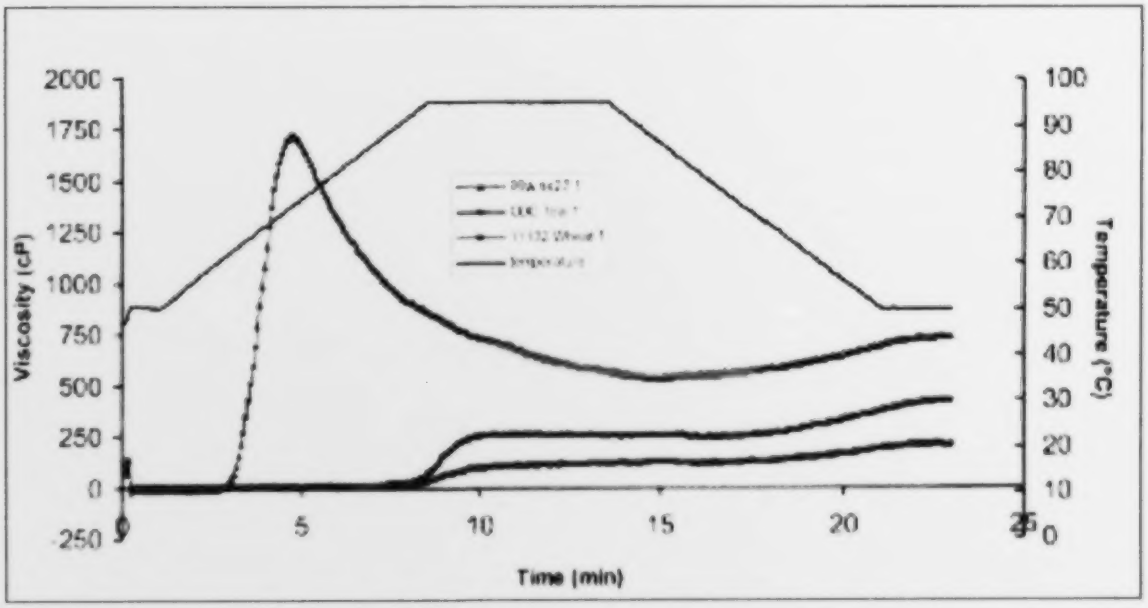


Figure 7. Pasting characteristics of the three starch samples.

Table 4: Differential Scanning Calorimetry (DSC) properties of starch extracted from normal, high amylose line and waxy wheat.

Starch Source	Endothermic Transition Temperatures (°C)[1]				Enthalpy of Gelatinization (ΔH J/g)
	Onset (T_0)	Peak (T_p)	Completion (T_c)	Range ($T_c - T_0$)	
CDC Teal	58.2±0.49[a]	63.23±0.29[d]	72.07±0.42[g]	13.87	9.93±0.02[j]
11132	52.67±0.12[b]	57.03±0.21[e]	65.37±0.12[h]	12.70	9.20±0.05[k]
99WAX27	54.46±0.17[c]	59.78±0.08[f]	74.3±0.16[i]	19.84	10.72±0.12[l]

[1] All data reported is on dry weight basis and represent the mean ± SD of three determinations. Means within a column with different superscripts are significantly different ($p<0.05$).

Conclusions: The results obtained in this project show that a deficiency in SSII can result in elevated amylose concentration. Several lines with SSII deficiency in the A or B or both A and B genomes were developed. Detailed analysis on one selected line 11132 shows: (i) higher apparent and total amylose performed by two independent laboratories; (ii) reduced starch swelling factor as compared to normal and waxy wheat starch; (iii) increased amylose leaching as compared to normal wheat starch; (iv) lower energy of enthalpy in gelatinization assays as compared to normal and waxy wheat starch. All these observations followed the trend that waxy wheat 99WAX27 and 11132 are on either side of normal wheat starch. These observations in combination with determination of amylose concentration by two independent laboratories using different techniques confirms that the wheat line 11132 has increased amylose concentration. However, the inability in this project to increase the amylose concentration beyond 32% suggests that SSII is not the only determinant affecting amylose concentration in wheat as reported earlier (Yamamori et al 2000). Recently, using a transgenic approach RNAi inhibition of starch branching enzyme genes resulted in wheat transformants with 70% amylose (regina et al., 2006). To avoid using a transgenic approach, further studies need to be carried out to screen natural variation for starch branching enzyme genes. Starch branching enzymes from the three wheat genomes are very similar in molecular mass and charge and difficult to separate by SDS-PAGE. Developing gene specific markers to screen global germplasm collection or a wheat TILLING population may be the only alternative to develop wheat lines with high amylose concentrations (≥40). With this approach, it may be possible to increase amylose concentrations beyond forty or fifty percent.

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Staff employed

Mr. Aman Kapoor – November 1, 2003 to May 31, 2004

Dr. Vinesh Verma – February 1, 2004 to October 31, 2005

(e) **Equipment** None

(f) Project Developed Materials

Wheat lines with elevated starch concentration are in various stages of development and evaluation.

(g) **Project Photos - See above**
Expense Statement

Will be sent by the University of Saskatchewan financial reporting.